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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Silvia Matthias P.A.	Pastorekova Ebert	Bratislava, Slovak Republic Magdeburg, Germany

☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

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TITLE OF THE INVENTION (280 characters max)

CAIX in Gastric Cancer

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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☒ Firm or Individual Name	Leona L. Lauder				
Address	465 California Street, Suite 450				
Address					
City	San Francisco	State	CA	ZIP	94104-1840
Country	United States	Telephone	415-981-2034	Fax	415-981-0332

ENCLOSED APPLICATION PARTS *(check all that apply)*

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Respectfully submitted,

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Date 10/16/03

REGISTRATION NO. (*if appropriate*) 30,863

Docket Number: MST-2390

TYPED or PRINTED NAME Leona L. Lauder

TELEPHONE 415-981-2034

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Silvia Pastorekova and Matthias P.A. Ebert

Serial No.: Group Art Unit:

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For : CAIX in Gastric Cancer

CERTIFICATION UNDER 37 CFR 1.10

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I hereby certify that the enclosed papers relating to the filing of a provisional application are being deposited with the United States Postal Service on October 16, 2003 in an envelope with sufficient postage for Express Mailing with "Express Mail Post Office to Addressee" Mailing Label Number EV 334343714 US addressed to: MAIL STOP PROVISIONAL PATENT APPLICATION, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Respectfully submitted,

Denise Ortega

Dated: October 16, 2003

CAIX in Gastric Cancer

This provisional application includes the accompanying manuscript -- Chen et al., "Carbonic Anhydrase IX (CA9) Expression in Gastric Cancer is Associated with Enhanced Invasion and Poor Prognosis." That manuscript, a copy of which follows, and the references cited therein are hereby incorporated by reference.

Carbonic Anhydrase IX (CA9) Expression in Gastric Cancer is Associated with Enhanced Invasion and Poor Prognosis

Running title: CA9 in gastric cancer

Jie Chen[1], Christoph Röcken[2], Juliane Hoffmann[1], Sabine Krüger[2], Uwe Lendeckel[3], Alba Rocco[1], Silvia Pastorekova[4], Peter Malfertheiner[1], Matthias P.A. Ebert[1]

[1]Department of Gastroenterology, Hepatology and Infectious Diseases, [2]Institute of Pathology, and [3]Institute of Experimental Internal Medicine, Otto-von-Guericke University, Magdeburg, Germany; [4]Institute of Virology, Slovak Academy of Sciences, 84246 Bratislava, Slovak Republic

Keywords: carboanhydrase, CA9, MN, gastric cancer, invasion, prognosis

Address for correspondence:
Matthias P.A. Ebert, MD
Department of Gastroenterology, Hepatology and Infectious Diseases
Otto-von-Guericke University
Leipzigerstr. 44
D-39120 Magdeburg, Germany
Tel: +49-391-6713156, Fax: +49-391-67190054
Email: Matthias.Ebert@medizin.uni-magdeburg.de

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Abstract

Background: Carbonic anhydrase IX (MN/CA9) catalyzes the reversible metabolism of carbon dioxide to carbonic acid, and has also been linked to malignant transformation and hypoxia in various cancers. While CA9 overexpression is frequently found in transformed cell lines and in several human malignancies, the role of CA9 in the pathogenesis of gastric cancer is less well known. *Methods:* Using gastric cancer tissues obtained from patients undergoing gastric cancer surgery and gastric cancer cell lines, we studied the expression of CA9 in gastric cancers by Western blot analysis, immunohistochemistry and realtime quantitative PCR. To analyse the invasive and growth promoting effect of CA9, AGS gastric cancer cells were transfected with CA9 cDNA. Gastric cancer cell lines were also treated with 5′-azadeoxycytidine to analyse methylation as a potential molecular mechanism underlying the loss of CA9 expression. *Results:* In the non-cancerous tissues CA9 was strongly expressed with membranous localization. In contrast, CA9 expression was frequently lost in diffuse- and intestinal-type gastric cancers ($p<0.001$). Reduction of CA9 expression in gastric cancer tissues and cell lines was confirmed by Western blot analysis and quantitative PCR analysis ($p<0.05$). However, gastric cancers that retained CA9 expression in the cancer cells, which was typically located at the invasion front of the cancers, exhibited a shorter post-operative survival ($p=0.028$). *In vitro* analysis revealed that the loss or reduced CA9 expression in gastric cancer cell lines was restored after treatment with 5′-azadeoxycytidine and was associated with increased invasion ($p<0.01$). Moreover, AGS cells transfected with CA9 exhibited significantly enhanced invasive growth and increased cell proliferation ($p<0.05$). *Conclusions:* While the loss of CA9 expression is frequent in gastric cancers, a subgroup of gastric cancers retain CA9 expression in the cancer cells at the invasion front which is associated with poor prognosis. While the loss of CA9 expression is regulated in part by methylation, reexpression of CA9 expression by inhibition of methylation as well as CA9 transfection is associated with increased invasion, supporting the hypothesis that increased CA9 expression may contribute to invasion, und thus advanced disease and tumor progression in a subset of gastric cancers.

Introduction

The carbonic anhydrase family includes eleven zinc metalloenzymes involved in reversible hydration-dehydration of carbone dioxide: $CO_2 + H_20 \Leftrightarrow HCO_3^- + H^+$. These molecules participate in a variety of physiological and biological processes and show remarkable diversity in tissue distribution, subcellular localization, and biological functions (1-3). Carbonic anhydrase IX, CA9, is one of the most recently identified isoenzymes (4,5). Because of the CA9 overexpression in transformed cell lines and in several human malignancies, it has been recognized as a tumor-associated antigen and linked to the development of human cancers (6-8). CA9 is a transmembranous glycoprotein with homology to the basic-helix-loop-helix domain proteins. Through transfection studies it has been demonstrated that CA9 can induce the transformation of 3T3 cells (4,5). Furthermore, recent studies have revealed that CA9 not only participates in the cell-cell or cell-matrix interaction, but also can be induced in hypoxia through the HIF-1 protein binding to the hypoxia-responsive element of the CA9 promoter (9). In various cancers, including renal cell cancer, colon cancer and lung cancer, CA9 expression is increased and correlates with the microvessel density and the levels of hypoxia in the tumors (6,9,10). Furthermore, lung and cervical cancers overexpress CA9, which is associated with a poor prognosis in these patients (7,11). However, CA9 has also been found in the gastric mucosa, which, together with the small intestine, presents the only normal human tissue expressing this protein (12,13). Different from non-gastric tumors, CA9 has been reported to be absent or reduced in gastric carcinoma cell lines and in primary gastric tumors (12,13). Interestingly, CA9 deficient mice develop gastric hyperplasia which is associated with increased proliferation (14), raising the question, whether the putative pathophysiological role of CA9 in gastric cancer development and progression is different from the one observed in cancers of non-gastric origin. The aim of this study was to analyse the expression of CA9 in the normal gastric mucosa and gastric cancers, to assess the prognostic significance of CA9 expression in gastric cancer and the biological effects associated with CA9 transfection and the reexpression of CA9 by inhibition of methylation in gastric cancer cell lines.

Materials and methods

Subjects. Tumorous and corresponding non-tumorous paraffin embedded tissue specimens from 59 patients (20 female, 39 male, age range 41-84 years) were retrieved from the archive of the Institute of Pathology of the University of Magdeburg for immunohistochemical analyses. 27 patients had suffered from diffuse type and 32 from intestinal type gastric cancer, according to the Lauren classification (15). For molecular analyses gastric cancer and corresponding non-lesional tissue were obtained immediately after surgery from 18 patients with gastric cancer (2 female, 16 male, age range 43-82 years). Tissue samples were snap-frozen in liquid nitrogen and stored at –80°C and further processed as described below, or fixed in 10% neutralized formalin and embedded in paraffin for histological processing. The study was approved by the Human Subjects Committee of the University of Magdeburg, Germany.

Histology. Formalin fixed, paraffin-embedded tissues were stained with haematoxylin and eosin. Gastric cancers were classified histologically as intestinal-type or diffuse-type according to the Lauren classification (15).

Cell lines. The gastric cancer cell lines MKN45, MKN28, AGS, N87 and the Hela cells were obtained from Riken Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (ATCC, Rockville, MD). All cell lines, except AGS and Hela cells, were maintained in RPMI medium (Gibco BRL, Rockville, MD, USA) with 10% fetal bovine serum. The AGS cell line was kept in F-12K medium with 10% fetal bovine serum and the Hela cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum.

Transient transfection assay. AGS gastric cancer cells were seeded at a density of 2×10^5 cells/60mm dish. Twenty-four hours later cells were transfected with a pSG5C vector (5µg) containing the human CA9 cDNA (1.5 kb, KpnI/SacI site) (kindly provided by Dr. S. Pastorekova, ref. 4), or an empty pCMVβ vector (control A) or were incubated with the Transfectam reagent alone (control B) (Promega, Mannheim) according to the manufacturer's recommendations with the optimal volume/weight ratio of Transfectam Reagent/DNA of 2µl/µg DNA. Protein expression was confirmed after 24h, 48h and 72h by Western blot analysis (not shown).

Treatment of cells with 5-aza-2'-deoxycytidine (5-aza-dC). Cells were seeded at a density of 1 x 10^6 cells/60mm dish. Twenty-four hours later, cells were treated with 5 μM 5-aza-dC (Sigma Chemical Co., Deisenhofen, Germany). The same concentration of DMSO was also used as a control for nonspecific solvent effect on cells. Total cellular protein was isolated 3 days after addition of 5-aza-dC as previously described (16).

Cell proliferation assay. AGS cells were grown in media supplemented with 10 % fetal calf serum (Gibco Invitrogen) and 50 μg/ml rifobacin. Parental AGS cells, AGS cells transfected with the pCMVβ vector and CA9 transfected AGS cells were seeded in 96 well plates at a density of 30.000 cells/200 μl/well. After 40 hrs of culture at 37°C, 5 % (v/v) CO_2, cells were pulsed for an additional 8 hrs with 3H-methyl-thymidine (0.2 μCi/well), and harvested onto glass fibre membranes. The incorporated radioactivity was measured by scintillation counting. In each case DNA synthesis was assessed 6 times in parallel and repeated once, resulting in a total of 12 experiments per cell line (17).

***In vitro* invasion assay.** Cellular invasion of AGS cells treated with DMSO or 5'-azadeoxycytidine was evaluated in 24-well Transwell chambers (Costar, Bodenheim, Germany) as described previously (18). The upper and lower culture compartments were separated by polycarbonate filters with 8μm pore size. Prior to invasion assays, the polycarbonate filter was coated with 100 ng matrigel matrix. For invasion assays, 3 x 10^4 cells per well were incubated on the reconstituted basement membrane for 72 hrs. Cells passing the filters and attaching to the lower sites of matrigel-coated membranes were harvested using trypsin/EDTA; the cell number was quantified in a Coulter Counter ZII (Coulter Immunotech, Marseille, France). The number of migrating cells was calculated from controls grown under identical culture conditions for 72 hrs in 24 well plates. All experiments were performed in triplicate.

Real-time quantitative analysis of CA9 mRNA levels. Tissue specimens were homogenized with an ultrasound homogenizer (Ultra-Turrax T25 basic, IKA, Staufen, Germany). After the removal of chromosomal DNA by DNase (Invitrogen, Karlsruhe, Germany), total RNA was extracted by using RNeasy Midikit (Qiagen, Hilden, Germany) and quantified by measuring the optical density at 260 nm and separated by gel electrophoresis. Total RNA (1 μg) was reverse transcribed at 37°C for 1 hr in a final volume of 20 μl reverse transcription buffer (50 mM Tris-HCl pH 8.3, 7 mM $MgCl_2$ and 40 mM KCl and 10 mM DTT) containing 100 U MMLV reverse

transcriptase, Rnase H Minus, Point Mutant (Promega, Mannheim, Germany), 16 U RNase inhibitors (Promega), 200 pmol random primer (Promega) and 0.5 mM dNTPs (Biomol Feinchemikalien, Hamburg, Germany). The reaction was terminated by incubating the mixture at 95°C for 5 min. PCR amplification of the cDNA was performed as previously described (17). Briefly, PCR primers were designed to amplify a 240 bp cDNA fragment of the CA9 gene (sense 5'-AGGAGGATCTGCCCAGTGA -3'; antisense 5'-GCCAATGACTCTGG TCATC -3') (5). The expression level of CA9 was determined by using the LightCycler technique (Roche Diagnostics GmbH, Mannheim, Germany). Standard curves were obtained by serial dilutions of at least three plasmid-DNA samples with the cloned PCR-fragment (TOPO TA Cloning kit, Invitrogen, The Netherlands) in each run. The housekeeping gene β2-microglobulin was chosen as a reference and each PCR result was normalised against β2-microglobulin. PCR analyses for CA9 were performed in 20 µl volumes in glass capillaries using the LightCycler DNA Master Hybridization Probes kit (Roche Diagnostics GmbH, Mannheim, Germany) in combination with Hybridization Probes (TIB MOLBIOL, Berlin, Germany). Hybridization Probes consist of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of PCR cycles. One probe is labeled at the 5'-end with a LightCycler Red fluorophore and the other probe is labeled at the 3'-end with fluorescein. 0.5 µM of each primer, 3 mM $MgCl_2$, 0.8 µM LightCycler Red 640 labeled Probe (5'-LC Red640-TTGAGGCTCCTGGAGATCCTCAp-3') and 0.4 µM Donor-F Probe (5'-CTGAAGTTAGAGGATCTACCTACTX-3') were used in each PCR run under the following conditions: initial denaturation at 95°C for 10 s followed by 45 cycles with denaturation at 95°C for 10 s, annealing at 60°C for 10 s and elongation at 72°C for 10 s, with a temperature transition rate of 20 °C s^{-1}. The Second Derivative Maximum method provided by the LightCycler software was used to estimate the concentration of each sample (17).

Immunohistochemistry. Deparaffinized serial sections were cut at 3 µm for immunohistochemistry and placed on Superfrost Plus glass slides. Immunostaining was performed with a monoclonal antibody directed against CA9, kindly provided by Dr. Pastorekova (12). For immunostaining, sections were deparaffinized in xylene and rehydrated in an alcohol series. Anti-CA9 (dilution 1:10) was administered for 1 h at 37 °C in a moist chamber, followed by incubation with biotinilated anti-mouse IgG/anti-rabbit IgG (1:200; Vector Laboratories; distributed by Camon, Wiesbaden, Germany) and ABC alkaline phosphatase reagent, each for 30 min at room temperature. Between steps the sections were washed in Tris buffered saline (TBS). Immunoreactions were visualized with the avidin biotin complex method

applying a Vectastain ABC alkaline phosphatase kit (distributed by Camon, Wiesbaden, Germany). Neufuchsin served as chromogen. All specimens were counterstained with hematoxylin. Primary antibodies were omitted for negative controls.

Evaluation of immunohistochemical results. A numerical scoring system with two categories was used to assess the observed expression of CA9 in tumor cells and gastric epithelium. Category A documented the number of immunoreactive cells as 0 (no immunoreactive cells), 1 (<10%), 2 (11 to 50%), and 3 (>50%). A positive case was defined as having a Category A value of 1. Category B documented the intensity of the immunostaining as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). Finally, the values for Category A and B were added to give the „immunoreactivity score" (IRS), which could range from 0 to 6. Note that the method of calculating the IRS does not allow the individual categories to add up to an IRS of 1.

Western blot analysis. Human gastric tissues and cell lines were lysed in a buffer containing 1 mM EDTA, 50 mM β-glycerophosphate, 2 mM sodium orthovanadate, 1% Triton-100, 10% glycerol, 1 mM DTT and protease inhibitors (10 mg/ml benzamidine, 2 mg/ml antipain, and 1 mg/ml leupeptin). The protein concentration of the supernatants was determined by the BCA assay. Twenty-five μg protein of each sample was adjusted to Laemmli buffer [2% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), 100 mM DTT, and 0.1% bromphenol blue], denatured by heating at 95°C for 5 min, and subsequently separated on 12% polyacrycamide gels by gel electrophoresis. After separation, proteins were electroblotted polyvinylidene difluoride membranes (Bio-Rad). The membrane was blocked in 5% non-fat milk in 1% TBST overnight, and then incubated with 1:200 anti-CA9 antibody for 1hr at room temperature. Membranes were washed three times in TBS/0.1% Tween 20, incubated for 1 h with a secondary antibody (1:2000) diluted in blocking solution. Membrane-bound secondary antibodies were detected by enhanced chemiluminescence following the instructions of the manufacturer. To ensure equal loading amounts, the blots were stripped in 200 mmol/L glycine, 1% Tween-20, 0.1% SDS, pH 2.2, for 2 hrs at room temperature and rehybridized using a monoclonal anti-β-actin antibody (dilution 1:2000; clone AC-74; Sigma) (16).

Statistical analysis. The number of proliferating/invading cells and the expression of CA9 was analysed using student's t test. The survival curve was plotted using the Kaplan-Meier method, and comparison of survival times was performed with the log-rank test. A p value < 0.05 was taken as the level of significance.

Results

Localization of CA9 expression in gastric cancer cells

The distribution pattern of CA9 was first investigated by immunohistochemistry (Fig. 1). CA9 was found in non-neoplastic gastric mucosa of every patient studied. It was confined to foveolar epithelial cells, fundic and antral glands (Fig. 1). Intestinal metaplasia was observed in 15 (26.3%) patients and CA9 was expressed at the brush border of the intestinal metaplasia in 10 patients (66.7%). CA9 was expressed in gastric cancer cells of 31 (54.0%) patients. No differences were found between intestinal and diffuse type of gastric cancer. CA9 was present in the tumor cells of 14 (51.9%) patients with diffuse type gastric cancer, in 12 (57.1%) patients with moderately differentiated intestinal type gastric cancer and 5 (55.6%) patients with poorly differentiated intestinal type gastric cancer. The mean total immunoreactivity score (IRS) for CA9 was calculated to be 1.95±1.98 for gastric cancer compared with 5.66±0.78 in the foveolar epithelium ($p<0.001$). Again no differences were found between diffuse or intestinal type gastric cancers. Table 1 summarizes the total immunoreactivity scores for CA9.

Quantitative analysis of CA9 expression in gastric cancers

CA9 mRNA and protein expression were then assessed by realtime quantitative PCR and Western blotting. Tumor samples were obtained from 18 patients with gastric cancer and matched corresponding non-neoplastic gastric mucosa was also available from these patients. CA9 mRNA levels in cancer and non-cancer tissues were assessed in 10 patients, whereas Western blot analysis was performed in 12 cases. In 5 cases, both Western blot analysis and realtime quantitative PCR was performed in the same patient, allowing a direct comparison of the expression levels of CA9 protein and mRNA in gastric cancer and non-neoplastic gastric mucosa. Overall the levels of CA9 protein and mRNA were significantly decreased in gastric cancers compared to the matched non-neoplastic mucosa ($p=0.04$). The direct comparison of 5 cases in which both CA9 protein and mRNA levels were assessed, revealed that in all cases reduced protein levels were associated by decreased CA9 mRNA levels (Fig. 2).

Prognostic significance of CA9 expression in gastric cancer

Survival data were obtained from 23 patients with gastric cancer undergoing gastric cancer resection. According to the immunohistochemical score as outlined above two groups of patients were classified as group A with low CA9 expression (IRS ≤ 3) versus group B with high CA9 expression in the cancer cells (IRS > 3). Post-operative survival time for patients with high CA9 expression was significantly shorter than in patients without or low CA9 expression ($p=0.0281$) (Fig. 3). Interestingly, expression of CA9 was very prominent at the site of infiltration of the muscularis propria, indicating that despite the overall loss of CA9 expression in gastric cancer, the re-expression of CA9 at the invasion front may contribute to the overall poor survival in patients with increased CA9 expression (Fig. 1).

CA9 transfection induces invasion and proliferation of AGS cells

CA9 mRNA and protein-levels were investigated in AGS, N87 and MKN28 gastric cancer cells by realtime PCR and Western blotting. HeLa cells served as positive control. CA9 mRNA and protein were found in N87 and MKN28 cells, albeit at significantly lower levels compared to HeLa cells (Fig. 2). CA9 mRNA and protein were undetectable in AGS cells, which were then chosen for transfection of CA9 cDNA in order to assess the biological changes associated with CA9 expression. AGS cells were transfected with full-length CA9 cDNA, with an empty expression vector (control B) or were treated only with the transfectam reagent without DNA transfer (control A). The expression of CA9 in transfected cells was confirmed by Western bloting (not shown). The invasive capability of transfected AGS cells was assessed using 24-well Transwell chambers. The expression of CA9 in AGS cells resulted in a significant increase of migrating cells compared with controls, i.e. AGS cells transfected with empty vector or incubation of parental AGS cells with transfectam only (controls A and B) (Fig. 4). Furthermore, transfected AGS cells showed a significant increase in cell proliferation compared with the two control groups (Fig. 4).

Biological effects of restoration of CA9 expression in gastric cancer cells by inhibition of methylation

The levels of CA9 mRNA were also analyzed in N87, MKN28, MKN45 and AGS cells after treatment with 5′-azadeoxycytidine, a demethylating agent. Treatment with 5′-azadeoxycytidine increased CA9 mRNA levels more than 5fold in N87, MKN45 and AGS cells, indicating that the expression of CA9 is, at least in part, regulated by methylation. No effect was observed in MNK28 cells (Fig. 5). The *in vitro* matrigel invasion assay was used to assess the invasive potential of 5′-azadeoxycytidine treated AGS cells compared to untreated AGS cells. Untreated AGS cells exhibited no striking difference in invasiveness as compared to DMSO treated AGS cells (control). In contrast, 2.9% of 5′-azadeoxycytidine treated AGS cells passed the reconstituted matrigel matrix, while only 1.05% of untreated and 1.04% of DMSO treated AGS cells were detectable on the lower side of the filters ($p<0.01$) (Fig. 5).

Discussion

Gastric cancer is the second most common cause of cancer-related deaths worldwide (19,20). Despite its decreasing incidence it remains a great challenge for clinicians and oncologists. In recent years various groups have analysed the genetic and molecular changes leading to gastric cancer. These changes include, among others, the overexpression of oncogenes, such as growth factor receptors *K-sam* and *c-met*, the loss of certain tumor suppressor genes, such as *APC* and *p53*, as well as alteration of adhesion molecules, including E-cadherin and the catenins (19-23). Recently, the group of carbonic anhydrases and especially CA9 have received increasing attention, since they have been linked to the process of malignant transformation and progression of various cancers (1-6). In gastric cancers, however, the expression is lost or down-regulated, indicating that the biological functions of CA9 must be more complex (12,13). Physiological analysis revealed that CA9 plays an important role in acid-base balance, ion exchange and the CO_2 transfer through the reversible dehydration of CO_2 and HCO_3^- (1). Furthermore, the transcription of the *CA9* gene is regulated by the von Hippel-Lindau tumor suppressor gene. The protein product of the von Hippel-Lindau tumor suppressor gene interacts with the ubiquitin ligase complex that is responsible for targeting HIF-1α for oxygen-dependent proteolysis (24,25). Thus, low levels of oxygen lead to stabilization of HIF-1α, which in turn leads to the increased expression of CA9 (24,25). Furthermore, areas of high expression of CA9 in cancers are linked to tumor hypoxia as reported in bladder and skin cancer and incubation of tumor cells under hypoxic conditions leads to the induction of CA9 expression (7-11).

While overexpression of CA9 has been reported in various cancers, including skin, bladder, colon and lung cancer, the expression is low or even lost in most gastric cancers (12,13). In our analysis CA9 expression was lost in the cancer cells in 31 of 57 cancers, while in the normal stomach expression of CA9 was retained in foveolar epithelial cells and in fundic and antral glands. A previous study by Pastorekova et al. assessed the expression of CA9 in a limited number of gastric cancers and also reported decreased CA9 expression in their gastric cancers (12). While loss of expression of CA9 could be interpreted as a consequence of the neoplastic changes, including dedifferentiation, taking place during gastric carcinogenesis, recent studies indicate that in fact this loss is not just an epiphenomenon but instead a critical change underlying the process of gastric carcinogenesis. This hypothesis is supported by the generation of CA9 deficient mice, in which the inactivation of the *CA9* gene leads to the development of gastric hyperplasia, which is associated with enhanced cellular proliferation (14). Together with

our analysis demonstrating loss of CA9 expression in approximately half of the gastric cancers, these studies in CA9 deficient mice indicate that CA9 may function as a critical differentiation factor in the stomach that also controls cell proliferation and growth of the gastric mucosa. Therefore, loss of CA9 expression as observed in the Western blot and PCR analysis may support the hypothesis that this loss of expression is critical for the development of gastric cancer and may be an early event in gastric carcinogenesis.

Our immunohistochemical analysis, however, revealed a further important observation. After applying an immunoreactivity score, two groups of CA9 expression patterns were identified in gastric cancer. Cancers expressing abundant CA9 exhibited a shorter post-operative survival compared to tumors with low levels of expression or no expression at all. A similar association of CA9 expression and poor prognosis has recently also been reported in non-small-cell lung cancers (11). In the further analysis of the immunohistochemical sections of gastric cancers that retained CA9 expression, we observed expression of CA9 primarily in cancer cells that were located at the invasion front of these cancers, indicating that while a loss of CA9 expression is a frequent event in gastric cancer, those tumors that retain CA9 expression exhibit increased invasiveness, which could contribute to their poor prognosis (26). These *in vivo* observations are supported by the *in vitro* analysis of CA9 overexpression in AGS gastric cancer cells. Upon transfection of CA9 cDNA in these gastric cancer cells, the cell proliferation and invasive growth of these cells was significantly enhanced. Thus, overexpression of CA9 in gastric cancer is also associated with enhanced cell proliferation and invasion, confirming the observation of CA9 expression at the invasion front of gastric cancers, which also exhibit a worse prognosis.

Recent studies in renal cancer indicate that CA9 expression is, at least in part, regulated by methylation of the CA9 gene promoter. Thus, hypomethylation of CpG at –74 bp and –6 bp sites in the CA9 promoter region is associated with increased CA9 expression in human renal cancer cell lines (27,28). Since we observed reduced or lost expression of CA9 in a large number of gastric cancers and gastric cancer cell lines, we treated 4 well established gastric cancer cell lines with 5'-azadeoxycytidine, a demethylating agent, in order to analyse whether this inhibition of methylation may lead to the restoration of CA9 expression. All cell lines, except for MKN28 cells, exhibited increased CA9 mRNA levels in the realtime PCR analysis after treatment with 5'-azadeoxycytidine, indicating that the expression of CA9 in gastric cancer cell lines is, at least in part, regulated by methylation of CpG sites. Since we observed a reexpression of CA9 in these cells after treating these cells with 5'-azadeoxycytdine we wanted to assess, whether this restoration of CA9 expression would also alter their biological characteristics. Therefore, these

cells were also analysed in an invasion assay that allows to assess changes in invasiveness of cells with and without treatment. While we observed no changes in invasiveness of AGS cells which were kept in media or DMSO added to the media, AGS cells incubated with 5'-azadeoxycytidine exhibited a significant, almost 3fold increased level of invasiveness, indicating that restoration of CA9 in these cells is associated with enhanced invasion.

From these data we assume that the loss of CA9 is an early event in gastric cancer, that may be associated with increased promoter methylation. Later in the process of gastric cancer progression CA9 expression is induced at the invasion front of the cancer cells which gives these cells an additional growth advantage by enhancing their proliferation and invasive growth. Inasmuch as HIF-1α is induced by intratumoral hypoxia, which in turn induces CA9 expression (24,25), it is assumable that the reexpression of CA9 at the invasion front of gastric cancers may result from the activation of the O_2-regulated subunit of HIF-1 leading to increased HIF-1α expression at the invasion front of gastric cancers, which has already been reported in colon and other cancers (29). In summary, while the frequent loss of CA9 expression observed in gastric cancer may be an early event, the overexpression of CA9 at the invasion front of a subset of gastric cancers may lead to invasive growth and thereby contributes to the growth and progression of this malignancy.

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Tables

Table 1. Expression of CA9 in gastric cancer as shown by immunohistochemistry.

Characteristics	Moderately differentiated intestinal type n=21	Poorly differentiated intestinal type n=9	Diffuse type n=27
Age (years±SD)	68.3±10.0	60.0±11.2	62.0±11.0
Gender (m/f)	15/6	7/2	15/12
IRS of cancer cells	1.67±1.73	2.05±1.99	1.96±2.10
IRS of foveolar epithelium	5.50±0.84	5.88±0.33	5.52±0.98
P-value*	<0.001	<0.001	<0.001

IRS denotes immunoreactivity score; * The P-value refers to the differences between the IRS of cancer cells and foveolar epithelium.

Figure legends

Figure 1. The distribution and expression pattern of CA9 was investigated by immunohistochemistry. Non-tumor and tumor were stained with anti-CA9 antibodies. CA9 was found in non-neoplastic gastric mucosa and significantly less often in gastric cancer. Note lack of immunostaining in gastric cancer of moderately (G2) differentiated intestinal type and diffuse type of gastric cancer, while non-neoplastic epithelial show strong immunostaining. Occasionally immunostaining was heterogeneous: the bottom set shows a poorly differentiated (G3) intestinal type gastric cancer with no immunostaining of the tumor cells in the mucosa and intense staining in a subset of tumor cells infiltrating the muscularis propria. Hematoxylin counterstain; Original magnification: x400.

Figure 2. A. Western blot analysis revealed reduced CA9 protein levels in gastric cancer (T) compared with non-neoplastic gastric mucosa (N). CA9 was identified as a 54 and 58 kDa isoform. β-actin protein levels were assessed for standardization of protein levels. No CA9 protein levels were identified in AGS cells, whereas low levels were found in N87 and MKN28 cells. Hela cells served as a control. **B.** Quantitative analysis of CA9 mRNA and protein levels in gastric tumors (T) as assessed by Western blot analysis and realtime PCR compared to the matched non-neoplastic gastric mucosa (N). In 5 cases protein and mRNA levels were assessed in both the cancerous and non-cancerous tissues and exhibited decreased levels in the cancerous part in all cases.

Figure 3. Survival analysis of patients with gastric cancer expressing low or high levels of the CA9 protein. Using an immunoreactivity score as outlined in 'Methods', a group of patients with a IRS ≤ 3 (CA9-) and a second group with a IRS > 3 (CA9+) were identified. Survival was significantly shorter in patients with increased CA9 expression (score > 3; CA9+) ($p=0.0281$).

Figure 4. A. Cellular invasion of AGS cells transfected with CA9 cDNA (CA9), incubated with the Transfectam reagent without DNA (control A) or transfected with the empty pCMVβ vector (control B) was evaluated in 24-well Transwell chambers (Costar, Bodenheim, Germany) as described above. The differences between AGS cells transfected with the empty pCMVβ vector and the CA9 transfected cells, as well as the cells without DNA transfer and the CA9 transfected cells were statistically significant (two-tailed, unpaired *t* test; mean ± SD). *Bars*, mean ± SD. **B.** Induction of cell proliferation by CA9 transfection in AGS cancer cells. Transfection of AGS

cells with CA9 cDNA led to a significant induction of cell proliferation compared to cells without DNA transfection (control A) or transfected with an empty pCMVβ vector (control B) (two-tailed, unpaired *t* test; mean ± SD). *Bars*, mean ± SD.

Figure 5. A. CA9 mRNA levels in gastric cancer cell lines were assessed with and without incubation with 5'-azadeoxycytidine. Basal mRNA expression (grey columns) was standardized in all cells and the relative changes after incubation with 5'-azadeoxycytidine (dark columns) was assessed by realtime PCR. While no significant change was observed for MKN28 cells, the other cells, i.e. AGS, MKN45 and N87 cells, exhibited a more than 5fold increase in CA9 mRNA levels following treatment with 5'-azadeoxycytidine. **B.** Cellular invasion of AGS cells treated with or without DMSO or 5'-azadeoxycytidine was evaluated in 24-well Transwell (8 μm pore size) chambers (Costar, Bodenheim, Germany). Invading cells were harvested from the lower side of the filters by using trypsin/EDTA. Cell number was quantified in a Coulter Counter ZII (Coulter Immunotech, Marseille, France). The differences between 5'-azadeoxycytidine (AZA) and untreated AGS cells (AGS, DMSO) was statistically significant (two-tailed, unpaired *t* test; $p<0.01$ (*); mean ± SD). *Bars*, mean ± SD.

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